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Research Article

PATHOLOGICAL INVESTIGATION OF AFRICAN SWINE FEVER DISEASE OUTBREAK IN PIG AT COMMERCIAL FARM SETTING

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ABSTRACT: African swine fever (ASF) is a devastating, contagious viral disease of swine. It is caused by the double stranded DNA virus of the *Asfivirus* genus belonging to the *Asfarviridae* family. The present study was conducted to investigate a disease outbreak in the month of March 2023 in an organized pig farm with a herd size of 400 animals in western Uttar Pradesh which exhibited clinical illness terminating with death. Mortality was noticed mostly in adult pigs including pregnant animals and the disease episode lasted for 20 days. On gross and histopathological examination, different visceral organs (spleen, kidney, lymph node and heart) showed haemorrhagic lesions. Lymphoid depletion was observed in white pulp of spleen and lymphatic nodules of lymph nodes. The main gross and histopathology lesions were characteristic of a viral haemorrhagic disease such as classical swine fever (CSF) or African swine fever (ASF). Eight clinical samples were found to be positive for ASFV. The tissue samples from necropsy animal were found to be positive for ASF virus and negative for CSF virus using PCR and reverse transcriptase- PCR (RT-PCR), respectively. Strong immunoreaction was observed in lung, spleen, heart and intestine. Based on pathological and molecular investigation, the etiology of the outbreak was confirmed as the African swine fever virus. The introduction of the disease in the herd may be due to unprocessed swill feeding sourced from hotels in Delhi and adjoining areas where previous ASF outbreaks were reported. Disease surveillance, health care and proper bio-security approaches are required to avoid the future spread of disease.

Keywords: African swine fever virus, Immunohistochemistry, Pathological investigation, Pig.

INTRODUCTION

African swine fever (ASF) is a devastating transboundary viral disease of pigs with high case fatality and mortality rate. It is caused by a large enveloped, linear dsDNA virus with icosahedral symmetry of the *Asfivirus* genus belonging to the *Asfarviridae* family [1]. The size of the genome is about 170-190 kb and has three regions including left and right variable regions (LVR and RVR) of 38-48 kb and 13-22 kb respectively and a long central conserved region (C-region) of about 125 kb. To date, 24 genotypes of the ASF virus have been reported globally, which are classified based on mainly the variation in the *B646L* gene C-terminal end which encodes the p72 capsid protein [2]. ASFV genotype II has been reported from India [3, 4]. The disease was first reported in Kenya in 1921 [5]. Later, the disease outbreaks were reported in various countries of the three continents, that is from Africa [6, 7], Europe [8, 9] and Asia [10, 11, 12]. In the recent year, India also faced several outbreaks of domestic and wild pigs [4, 13, 14]. The first outbreak occurred in Assam and Arunachal Pradesh in the year 2020 [13]. Subsequently, it was reported from other parts of the country including Andhra Pradesh, Himachal Pradesh, Gujarat, West Bengal, Tamil Nadu and Kerala [15, 16]. The epizootic of ASF has grievously impacted pig farming, causing numerous farmers to lose their entire herd, especially in the pig-rearing-dependent North-Eastern region of

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India [17]. The virus uses three different transmission cycles, including, tick (*Ornithodoros* genus) to the wild pig (sylvatic cycle), tick to domestic pig and pig-to-pig transmission [18]. All secretions and excretions of the infected animal, meat product, meat scraps and blood meal of the infected pigs can be a source of fresh infection [19].

The disease can be either per acute, acute, subacute or chronic based on the virulence of the viral isolate. The clinical signs in affected pigs vary from per acute death to unapparent clinical signs. Infection from virulent strain results in high fever, erythematous lesions on the skin, anorexia, vomiting, watery to bloody diarrhea, conjunctivitis, severe depression, epistaxis, incoordination and abortion in pregnant sows [20]. The epidemiology of the disease is complex and different epidemiological patterns have been documented across the globe. Presently, the country is facing ASF outbreaks in different farm settings as information on epidemiological aspects, pathology and biology of pathogens is very limited in the nation. Further, due to lack of potent commercial vaccines, early detection and depopulation are critical measures to minimise the spread in infections among domestic and wild pigs. This report focuses on the detection and description of histopathological lesions in pigs to enhance our understanding of pathology in commercial farm settings.

MATERIALS AND METHODS

Outbreak

An outbreak was investigated in an organized farm having 400 large white Yorkshire pigs located at the periphery of metropolitan cities. History revealed that the animals were maintained with hotel refuges and kitchen waste collected from metropolitan cities. The disease episode started in the month of March 2023. The infected animals showed clinical signs of depression, anorexia and dyspnoea without responding to routine symptomatic treatment followed by death. Within 10-15 days, 50% of the animals in the herd died. One representative dead adult sow with a clinical history of depression, anorexia and dyspnoea for 3 days before death was presented for necropsy examination at post-mortem facility, Division of Pathology, IVRI, Izatnagar, Bareilly. The clinical signs and mortality pattern of the herd were investigated thoroughly. Oral swabs (n=10) of farms from ill and apparently healthy animals were screened for molecular study.

Pathological examination and sampling

Detailed post-mortem examination was carried out and all findings were carefully recorded. Various tissue samples (Spleen, lung, heart, kidneys, lymph nodes, liver, skin, stomach, intestine, brain and pharyngeal tonsils) were collected and preserved in 10 percent neutral buffered formalin (10% NBF) till further processing. Also, some portion of tissue samples (spleen, lungs, lymph nodes, pharyngeal tonsils, heart, intestine, liver and kidneys) were collected on ice followed by preservation at -20º C for molecular investigation. Formalin-fixed tissues were processed routinely and paraffin- embedded sections were stained by H and E stain according to standard protocol [21].

Immunohistochemical detection of ASFV antigen in tissue

The formalin fixed paraffin-embedded tissue sections (Spleen, lung, heart, kidneys, lymph nodes, liver, intestine and pharyngeal tonsils) were taken on the poly-L lysine (0.1%) (Sigma P8920) coated glass slides. Coating of slide with poly-L-lysin Deparaffinized and rehydrated, sections were treated with proteinase K (20 µg/ml) and incubated for 15 min in a humid chamber at 37 ◦C. Immunohistochemistry was performed using rabbit anti-ASF virus hyper immune serum and secondary IgG-HRPO antibodies (REAL GENE, Germany) as per previously described procedures [22]. After mounting, the sections were examined under a light microscope.

Laboratory diagnosis

Swab (n=10) and tissue samples (n=8) were triturated in PBS and total nucleic acid was extracted using magnetic beads (ThermoFisher) as per the recommendations of the manufacturer. ASFV-specific genome was detected using conventional PCR following OIE recommendations. Briefly, forward primer, PPA1: AGTTATGGGAAACCCGACCC and reverse primer PPA2; CCCTGAATCGGAGCATCCT targeting the P72 protein-encoding gene of ASF [23] was used along with Taq Master Mix from Genes2ME. Amplification was carried out in a thermal cycler with initial denaturation at 95ºC for 3 min followed by 35 cycles of denaturation at 95ºC for 30 sec; annealing at 56ºC for 30 sec and extension at 72ºC for 30 sec.

The TRIzol® method was employed for RNA isolation from pooled tissue samples, and subsequent cDNA synthesis was performed using the high-capacity cDNA synthesis kit from Invitrogen. The synthesized cDNA was subjected to PCR amplification with previously published primers, forward primer, HCVgt1: CTGGGTGGTCTAAGTCCTGA and reverse

primer HCVgt4: TCAACTCCATGTGCCATGTA targeting 5' NTR region of CSF [24]. For amplification, thermal cyclic condition was 94ºC for 5 min for initial denaturation followed by 35 cycles of denaturation at 94ºC for 30 sec; annealing at 59ºC for 45 sec and extension at 72ºC for 45 sec and final extension at 72ºC for 7 min. The amplicons generated were resolved in 1% agarose gel.

RESULTS AND DISCUSSION

Clinical signs were first noticed in adult animals and the death was noticed within 5 days. Clinical signs included fever, anorexia, dullness, respiratory distress, vomiting, and coma followed by death. Mortality within herd occurred for a period of 20 days. Ten pregnant animals aborted and some showed mild clinical signs and few recovered. The clinical features and mortality pattern suggests that the disease is acute and contagious. The clinical signs in the affected pigs ranged from non-specific to fever, respiratory distress, depression, anorexia, vomition, erythematous lesions on skin and abortions in case of pregnant animals were similar to those reported previously in ASF infected pigs [3, 20, 25].

The disease outbreak in pigs was investigated to identify the etiology along with pathological and epidemiological aspects in organized farm settings. Upon detailed post-mortem investigation, externally, bluish-red irregular patchy areas on the skin at the perianal area, ventral surface of the abdomen, medial surface of both thigh and external pinna were noticed (Fig. 1. A and B). Large amount of blood-tinged fluid was found in both the peritoneal and thoracic cavities (Fig. 1. C and D). Grossly, the spleen showed severe congestion with 2-3 times enlargement (Fig. 2A), multifocal hemorrhagic lymphadenitis involving the majority of lymph nodes throughout the body with severe involvement of mandibular lymph nodes (Fig. 2B). The liver was diffusely congested (Fig. 2C). The heart showed multifocal pinpoint to coalescing hemorrhages on epicardium (Fig. 2D), and endocardium. Pharyngeal tonsils were highly congested. Diffuse petechial hemorrhages throughout the outer surface of the cortex of both the kidneys and on the longitudinal cut section both the cortex and medulla of kidneys were congested (Fig. 3A and B). Lungs were edematous with frothy fluid in airways with distinguished lobulations (Fig. 3C). Gastrointestinal mucosa was highly congested. Severe congestion of meningeal blood vessels (Fig. 3D) was noticed. Gross lesions include cutaneous erythemas, splenomegaly with haemorrhages, turkey egg appearance of kidneys, pulmonary edema. haemorrhages on the heart and lymphadenitis with haemorrhages involving multiple lymph nodes of the body were consistent with the previous reports from India and elsewhere [3, 14]. Haemorrhagic distended gall bladder was not noticed in the present case, which is one of the consistent lesions in pigs affected with ASFV [3, 14].

Further laboratory investigations were carried out by detailed histopathological examination of the tissues. Microscopically, the spleen showed an increased number of erythrocytes in red pulp and severe depletion of lymphoid tissue of white pulp with erythrocyte flooding the sinusoids (Fig. 4A and B). Lymph nodes showed severe lymphoid depletion and congestion of blood vessels (Fig. 4C and D). Lungs showed oedematous fluid-filled alveoli and

Table 1. Organ wise histopathological, molecular and immunohistochemical findings.

Organs	Findings		
	Key histopathological lesions	PCR for ASFV	IHC
Spleen	Lymphoid depletion in white pulp ymphoid necrosis	Positive	++
Lymph nodes	Lymphoid necrosis Haemorrhages	Positive	+
Tonsil	Diffused lymphoid follicles with mild necrosis	Positive	+
Lung	Pulmonary edema, Interstitial pneumonia	Positive	++
Liver	Multifocal haemorrhages	Positive	+
Heart	Fragmentations of cardiac muscles	Positive	++
Skin	Perivascular infiltration of mononuclear inflammatory cells in dermis.	NP	NP
Kidney	Tubular necrosis Interstitial haemorrhages	Positive	+
Stomach	Haemorrhages	NP	NP
Intestine	Haemorrhages	Positive	++
Brain	Congestion Perivascular cuffing	NP	NP

+ immunoreaction, ++ strong immunoreactions: NP = Not performed.

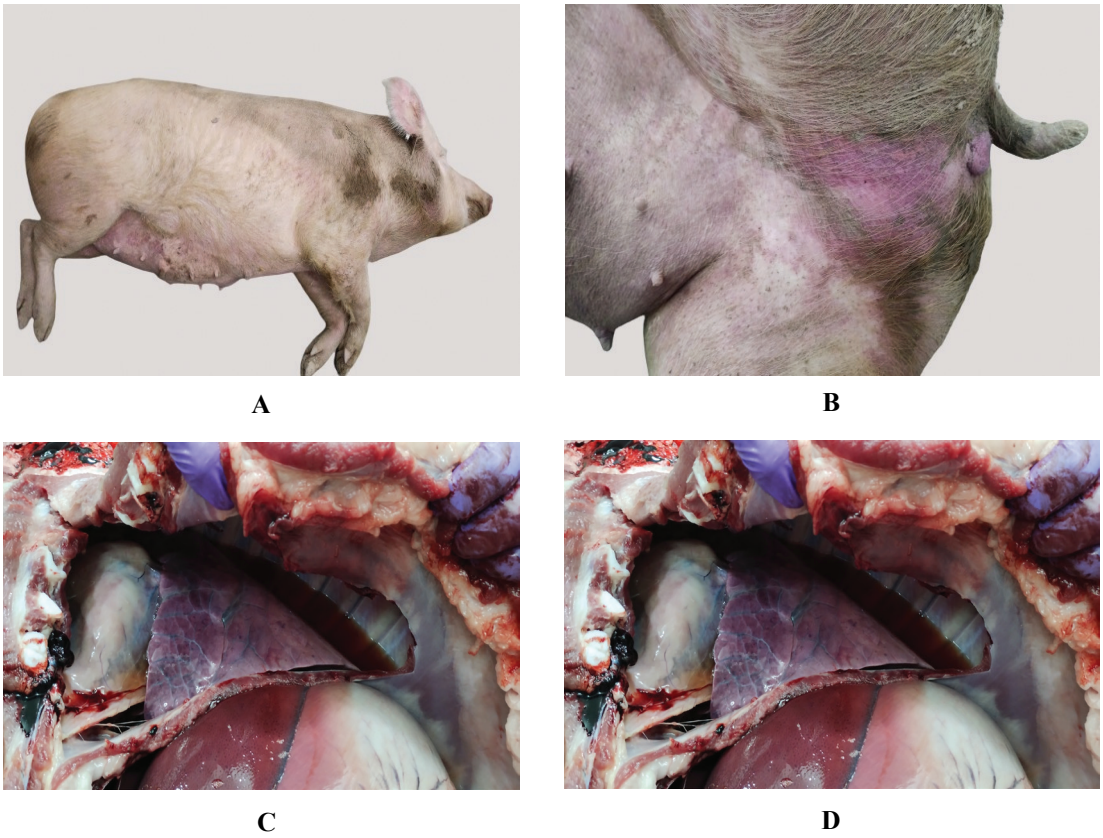

Fig. 1. Gross lesions of ASFV infected pig. Presence of erythematous patchy areas at lower abdomen (A) and perianal region (B); blood tinged fluid in thoracic cavity (C) and abdominal cavity (D).

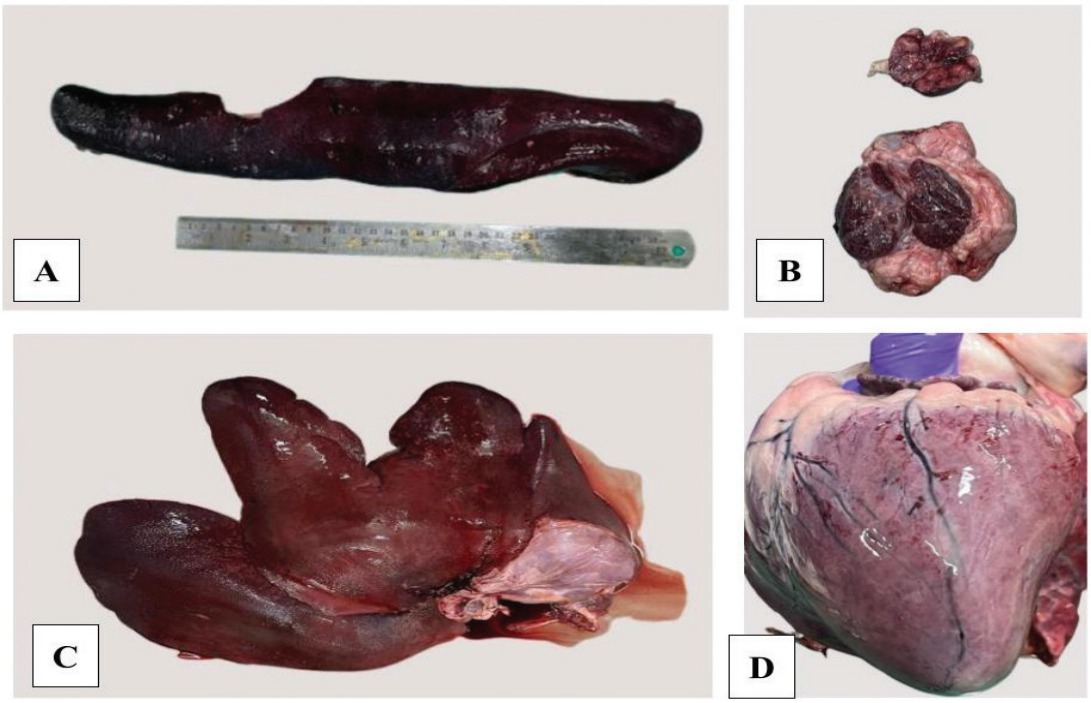

Fig. 2. Gross lesions of ASFV infected pig. [Enlarged and highly congested spleen with rounded borders (A); Enlarged lymph nodes with multiple pin-point haemorrhages (B); highly congested liver (C) and multiple pin-point to coalescing haemorrhages on epicardium of heart (D)].

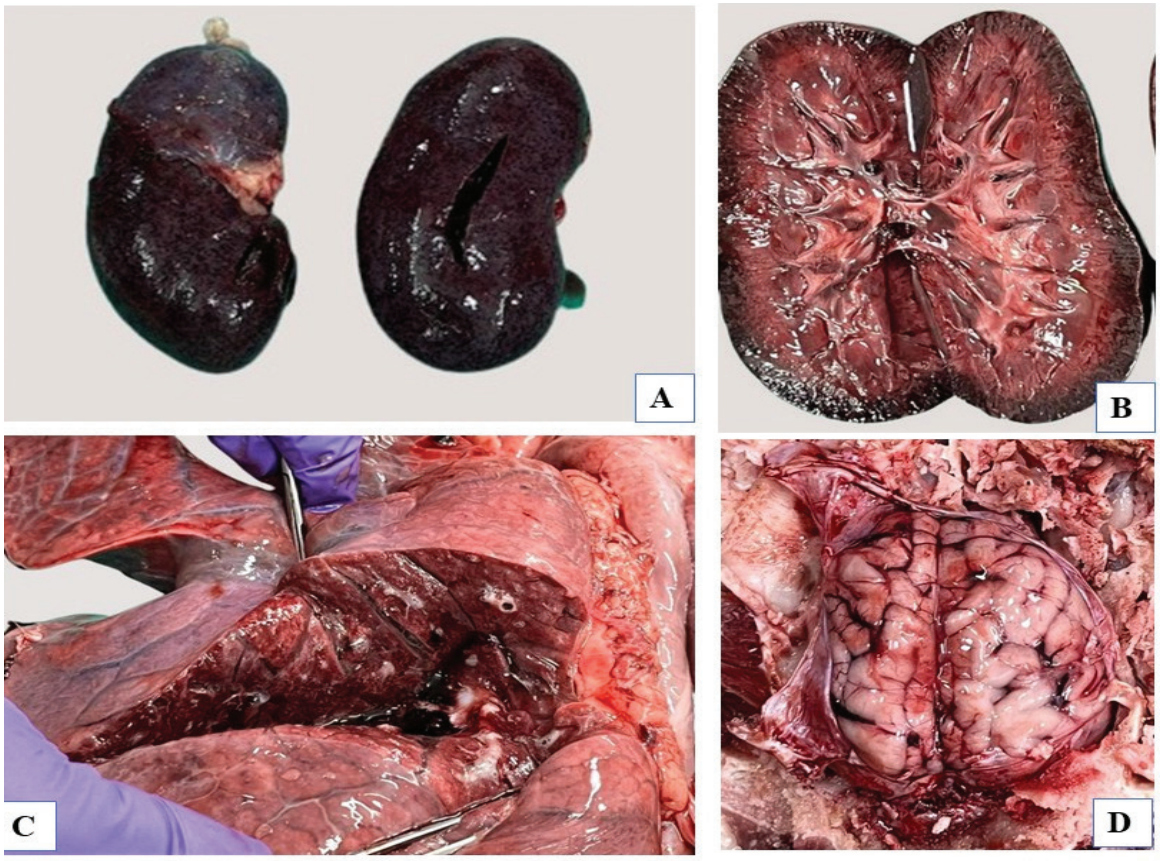

Fig. 3. Highly congested kidneys with diffuse, petechial haemorrhages on the renal cortex (A) and (B). Oedematous lung with frothy fluid in airways (C); Congestion of meningeal blood vessels with oedematous brain (D).

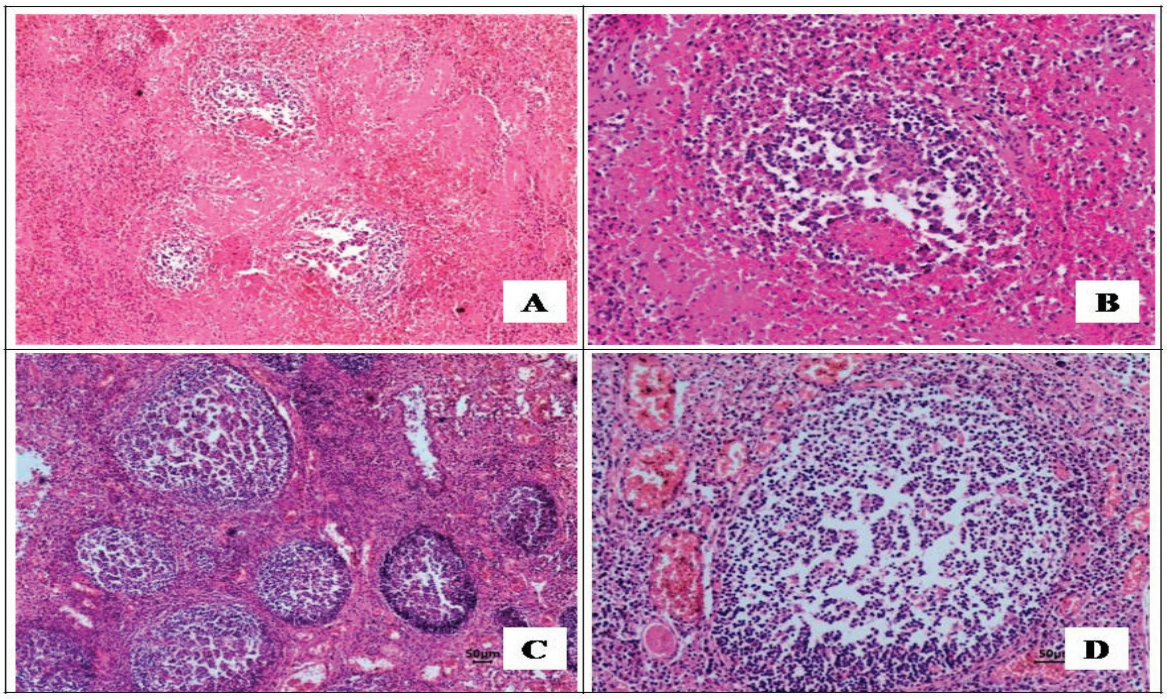

Fig. 4. Spleen and Lymph node. A. Red pulp filled with excess of erythrocytes and small lymphoid follicles in the white pulp, spleen, 4X, H and E. **B.** Lymphoid depletion in the lymphoid follicle with entry of erythrocytes, spleen, 10X, H and E. C and D. Lymphoid depletion in lymphoid follicles with congestion of blood vessels and capillaries, lymph node, 4X and 10X, respectively, H and E.

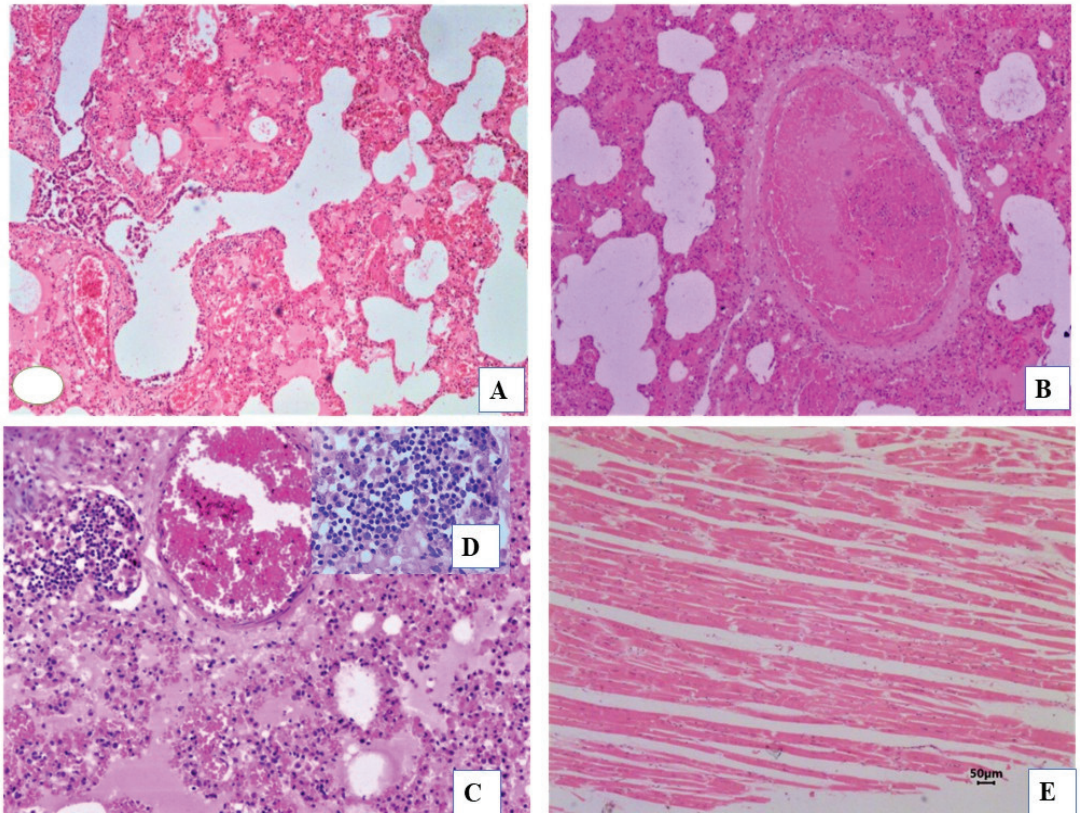

Fig. 5. Lung and Heart. A. Interstitial oedema and congestion pulmonary blood vessels, lung, 4X, H and E. B. Thrombosis in lung, 4X, H and E. C and D. Focal infiltration of mononuclear inflammatory cells around blood vessel, lung, 10X and 40X, respectively, H and E. E. Fragmentation of cardiac muscle fibres, Heart, 4X, H and E.

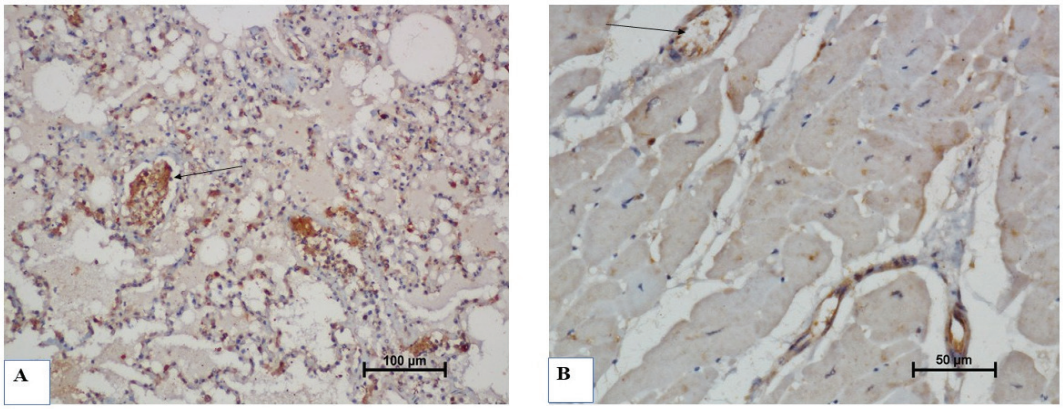

Fig. 6. ASFV antigen detection in tissues: Positive immunostaining for ASV antigen in lung pulmonary vessels and alveolar interstitium. IHC X20. (A). Positive immunostaining for ASV antigen in blood vessels of heart IHC X40 (B).

interstitium, focal mononuclear cell aggregations around pulmonary blood vessels, interstitial pneumonia, thrombus formations in multiple pulmonary blood vessels and desquamation of bronchiolar epithelium and congestion of blood vessels (Fig. 5. A, B, C and D). Liver showed focal haemorrhages. Haemorrhages were also notice in stomach and intestine. There was mild lymphocytic depletion of the Peyer's patches of intestine. In the heart, cardiac muscles showed fragmentations (Fig 5. E). The renal cortex showed interstitial hemorrhages, glomeruli showed hyperaemia with hypercellularity, degeneration and necrosis of renal tubules and the presence of proteinaceous casts within tubules. There was congestion of blood vessel and perivascular cuffing in brain. Hypertrophy of endothelial cells of blood vessels of skin and mononuclear cell infiltration mainly comprising of macrophages around the blood vessels of skin were noticed. Microscopic

lesions including splenic white pulp depletion, depletion of cells in lymphoid follicles of lymph nodes with congestion, degeneration and necrosis of renal tubular epithelial cells, interstitial haemorrhages in kidneys, pulmonary oedema with infiltration of mononuclear cells and degeneration and haemorrhages in cardiac muscles were observed similar to earlier reports from various countries including India ([4,25]. Antigen of ASFV was detected in pulmonary vessels and alveolar interstitium of lung (Fig. 6A) which was reported earlier as primary site [22, 26]. Strong immunoreactions were shown in spleen, intestine and heart (Fig. 6B)

The observed findings were most expected in one more devastating disease of pigs, that is, classical swine fever (CSF) [27, 28]. Hence, the samples were subjected to molecular assay for the diagnosis of above two similar diseases in pig. As the both viruses are high risk categories pathogens, samples from one representative animal were subjected for laboratory diagnosis. The confirmatory diagnosis for ASF was made using diagnostic primers. Conventional PCR following OIE recommendations resulted in a 257-base pair (bp) amplicon (Fig. 7). Out of 10 clinical samples 8 samples were found to be positive for ASFV and all tissue samples of various organs (n=8) were found to be positive for ASFV. The tissue samples (spleen, lymph nodes and lung) subjected to conventional RT-PCR was found to be negative for CSFV. The molecular assays confirmed the outbreak associated with ASFV.

In the presented study, the pigs were routinely fed with hotel wastes collected from several hotels and restaurants in Delhi and its surroundings where chances of contamination of pork and pork product with ASFV are high. This could be one of the most possible routes of introduction of the virus into the herd. The ASFV has crossed long distances within the countries and international borders as observed in outbreaks at European countries [18] and Korea [11]. Similarly, the deadly virus has spread recently over the pig-producing states of India since its first introduction. As there is no vaccine for ASF till date in India, the States and Union Territories free of such viral outbreaks, should focus on strict biosecurity and active surveillance to prevent the introduction of disease. Further human activities and emergency selling of apparently healthy pigs during disease episodes play a significant role in disease transmission. Such risk factors may also be considered in disease epidemiology in Indian farm settings.

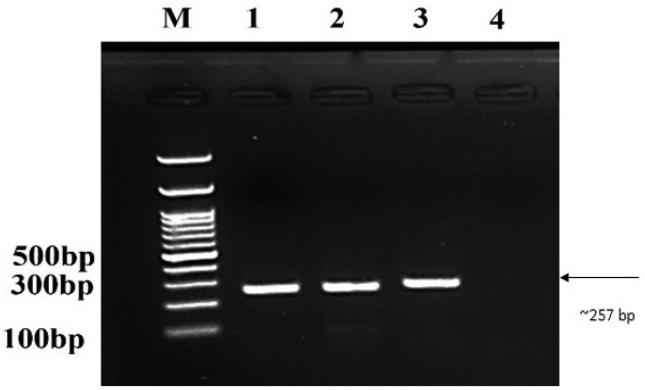

Fig. 7. Agarose gel electrophoresis of PCR amplicons of ASFV. **(**Lane M: Ladder 100bp, Lane 1: Spleen , Lane 2: Lymph node, Lane 3: Lungs and Lane 4: Negative control).

CONCLUSION

Based on history, clinical signs, pathological and molecular examination, the etiological agent causing the outbreak is confirmed as ASFV. In the present study, the source of entry of infection was suspected to be hotel refuges and kitchen waste collected from metropolitan cities where mixing of infected pork is one of the highest possibilities. This necessitates creating awareness among pig raisers regarding various possible risk factors associated with ASF disease transmission. Further, strict bio security measures, surveillance and health-care preparedness should be implemented by local animal health authorities to prevent the spread of such devastating disease.

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